Do the COL1A1 and *Taq 1* Vitamin D receptor polymorphisms have a role in identifying individuals at risk of developing osteoporosis?

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SUMMARY

The distribution of the *Taq 1* polymorphism in the vitamin D receptor (VDR) gene and the *MSc 1* polymorphism in the collagen 1 alpha 1 (COL 1AI) gene were studied in 266 female and 55 male patients attending an osteoporosis clinic. Allele frequency in control (T- or Z-score >-1.0) and osteoporotic (T- or Z-scores <-2.5) groups were compared using Chi squared tests. No differences were found between the 2 groups with either of the polymorphisms. When allele frequency was compared in patients with and without history of fracture, no differences were found in the frequency of the COL1A1 alleles. However there were significantly more fracture patients, who had been previously treated with corticosteroids for other conditions, carrying the T allele of the VDR polymorphism ($X^2 = 5.65$, p>0.01<0.02). In conclusion, neither of these polymorphisms aid in the prediction of osteoporosis but the VDR T allele may carry an increased fracture risk in patients who require corticosteroid treatment.

INTRODUCTION

Bone mineral density (BMD) is regulated by a complex interaction of genetic and environmental factors. Polymorphisms in the vitamin D receptor (VDR) and regulatory region of the type I collagen gene (COL1A1) have been associated with decreased bone mass and increased risk of osteoporotic fractures. Morrison[1] described a *Taq 1* polymorphism in codon 352 of exon 9 of the VDR gene resulting from a C→T transition. Grant[2] described a novel G→T polymorphism in the regulatory region of the COL1A1 gene which was found to be strongly associated with low BMD and increased risk of osteoporotic fracture.

We have studied the distribution of these two polymorphisms in a group of people referred to the osteoporosis clinic in Northern Ireland to assess if there is any association between either of these genotypes and low bone mineral density (BMD) or the risk of osteoporotic fracture in our local population.

METHODS

Patients

Patients attending the osteoporosis clinic for bone scans were studied. These patients had been referred to the clinic for a variety of different reasons e.g. family history, early menopause and long term treatment with corticosteroids. The bone density was measured by dual energy X-ray absorptiometry (DXA) using a Hologic4500A bone densitometer. Lumbar spine (L1-L4) and total hip BMD were measured and T-scores and Z-scores obtained. The local ethical committee approved the study and patients gave informed written consent. Blood was collected from 266 females (aged 28-83, median 64 years) and 55 males (aged 22-75, median 51 years). All subjects were Caucasian and 62 of the females (23%) and 10 of the males (18%) had a family history of osteoporosis. The BMD result from the subject's first visit was recorded and a blood sample sent for gene studies. At the end of the study (after the

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genotyping had been carried out) the patients were classified as to whether or not they had been treated with corticosteroids, for other medical conditions, and whether or not they had suffered a low trauma fracture. Steroid treatment was defined as ≥7.5mg for ≥6months and the classification of a low trauma fracture was a subjective assessment made by the doctor after discussion with the patient.

DNA Isolation

Ten millilitres of blood was collected from each patient into tubes containing EDTA. The plasma was removed and the DNA was extracted from the white cells using the method of Jean Pierre.[3] The DNA was dissolved in Tris-EDTA buffer (10mM) to give a concentration of approximately 250μg/mL and stored at −70°C until analysis. Before analysis the samples were diluted to 25μg/mL with double distilled, sterile water.

COL1A1 Genotyping

The primers used were those described by Grant,[2] namely a forward primer of 5'-TAACTTCTGGACTATTTGCGGACTTTTTGG-3' and a reverse primer of 5'-GTCCAGCCCTCATCCTGGCC-3'. The primers were purchased from Gibco BRL, Life Technologies, UK. Polymerase chain reaction (PCR) was carried out using Taq DNA polymerase (Boehringer Mannheim) under standard conditions on a Perkin Elmer 2400 Thermal Cycler. The thermal cycling protocol consisted of 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds and 72°C for 30 seconds with an initial denaturing step of 94°C for 3 minutes before the first cycle and a final extension step of 72°C for 7 minutes. The PCR product was incubated at 37°C with *Msc 1* overnight (17 hours) and the digests electrophoresed on a 1% agarose (Gibco

BRL, UK) plus 2.5% Nusieve (KMC Products, UK) gel and stained with ethidium bromide. Wild type DNA (G/G) has a single band at 255bp, heterozygotes for the G→T polymorphism have two bands at 237bp and 255bp, and homozygotes for T/T have one band at 237bp. These correspond to the SS, Ss and ss genotypes respectively.

Vitamin D Receptor Genotyping

The primers used were those described by Morrison[1] namely a forward primer of 5'-CAGAGCATGGACAGGGAGCAA-3' and a reverse primer of 5'-GCAACTCCTCATGGCTGAGGTCTC-3'. The primers were purchased from Gibco BRL, Life Technologies, UK. PCR was carried out using Taq DNA polymerase (Gibco BRL) using standard conditions on a Perkin Elmer 2400 Thermal Cycler. The protocol was 35 cycles of 94°C for 30 seconds, 63°C for 20 seconds and 72°C for 20 seconds with an initial denaturing step of 94°C for 3 minutes and a final extension step of 72°C for 7 minutes. The PCR product was digested with *Taq1* for 2 hours at 65°C, the digests electrophoresed on a 2% agarose gel and stained with ethidium bromide. Homozygotes for the T nucleotide (TT) have two bands at 495bp and 245bp, heterozygotes with C→T (Tt) have four bands at 495bp, 245bp, 290bp and 205bp and homozygotes for the C (tt) nucleotide have three bands at 290bp, 245bp and 205bp.

Statistics

The WHO criteria for defining patients with osteoporosis classifies those with T-score of >-1.0 as "normal", <-1.0->-2.5 as osteopenic and < -2.5 as osteoporotic. The T-score compares the BMD with that of the mean for a young adult population. Since our population spanned such a

TABLE I

Summary of genotypes

	No. of Patients	sstt f	ssTt f	ssTT f	Sstt f	SsTt f	SsTT f	SStt f	SSTt f	SSTT f
Total population	314	0.6	1.6	0.3	4.8	14.3	9.2	8.6	33.1	27.4
All Females	261	0.8	1.9	0.4	5.0	14.9	8.4	9.2	32.6	26.8
All Males	53	0.0	0.0	0.0	3.8	11.3	13.2	5.7	35.8	30.2

f = frequency (%)

TABLE II

Demographic details of patients, who have had no steroid treatment, when classified by lumbar and hip T- and Z-scores

	Females			Males		
	Osteoporotic	Osteopenic	Control	Osteoporotic	Osteopenic	Control
Lumbar T-score						
Number	125	48	29	12	9	4
Age Range (years)	35-83	28-76	34-78	29-65	28-75	41-70
Median age (years)	63	64	58	46	41	66
Lumbar Z-score						
Number	36	87	79	8	13	4
Age Range (years)	35-78	28-83	37-78	29-65	28-75	41-70
Median age (years)	64	63	64	52	44	66
Hip T-score						
Number	61	91	48	2	13	10
Age Range (years)	35-83	28-81	34-75	39-59	29-75	28-70
Median age (years)	67	64	58	49	46	45
Hip Z-score						
Number	11	72	117	1	11	13
Age Range (years)	35-76	28-83	34-81	39	29-75	28-70
Median age (years)	65	63	64	39	46	49

wide age group (28-83 years) we also classified patients according to their Z-scores which compares their BMD with age-matched controls. In order to be as comprehensive as possible we used both the T-score and Z-score at both the lumbar and hip sites to classify patients as those with normal bone density, those who were osteopenic, and those with osteoporosis. The numbers in each group varied according the site and scoring system used (Tables II and III). Those with T or Z scores >-1.0 were regarded as having normal bone density and were classified as our "Control" group. The normal (or control), osteopenic and osteoporotic groups were sorted according to whether or not they had received steroid therapy and whether or not they had sustained a low trauma fracture. The Chi-squared test was used to compare the frequency of the S with s and T with t alleles in patients who were classified as "controls" (T- or Z- score >-1.0) with those who were classified as "osteoporotic" (T- or Z- score <-2.5) using both the lumbar and hip BMO scans. Chi-squared was also used to compare the frequency of the S, s, T and t alleles in patients who had sustained a low trauma fracture with those who had no history of fracture.

Results

Fifty females and 22 males had received steroid therapy ($\geq$7.5mg for $\geq$6 months minimum). Thirteen females and seven males were uncertain as to whether or not they had received a significant course of steroids. These 20 patients were included in the calculation of the overall genotype frequency in the population (Table I) but were not included when the patients were divided into steroid and no steroid groups. There were no hip scores for five female patients and, due to technical difficulties with the assays, we were unable to obtain COL1A1 genotypes on five patient samples (4 females and 1 male) and VDR genotypes on two patient samples (1 female and 1 male).

The numbers of patients classified as controls, osteopenic and osteoporotic differed according to whether they were classified according to their T-score or Z-score or according to their lumbar or hip scores (Tables II and III).

The genotype frequency in our total population for the collagen *Spl* site ploymorphism was 69% SS, 28% Ss and 3% ss, which conforms to Hardy-Weinberg equilibrium. Females had the same distribution but none of the males in our study

TABLE III

Demographic details of steroid treated patients when classified by lumbar and hip T- and Z- scores

	Females			Males		
	Osteoporotic	*Osteopenic*	*Control*	*Osteoporotic*	*Osteopenic*	*Control*
Lumbar T-score						
Number	29	16	5	12	9	1
Age Range (years)	35-81	35-83	36-60	28-68	22-73	41
Median age (years)	65	67	44	47	54	41
Lumbar Z-score						
Number	7	17	26	10	9	3
Age Range (years)	50-61	35-74	36-83	28-66	22-73	41-61
Median age(years)	60	58	68	49	54	49
Hip T-score						
Number	14	20	13	4	15	3
Age Range (years)	50-81	39-83	35-76	38-64	22-73	41-61
Median age (years)	63	66	60	52	51	52
Hip Z-score						
Number	2	16	29	2	14	6
Age Range (years)	73-81	39-75	35-83	38-41	22-69	41-73
Median age (years)	77	59	68	40	53	53

had the ss genotype, with 72% being SS and 28% being Ss. The genotype frequency of the *Taq 1* polymorphism in our population is 37% TT, 49% Tt and 14% tt, which conforms to Hardy-Weinberg equilibrium. The frequency in the female population was 36% TT, 49% Tt and 15% tt and in the male population it was 43% TT, 47% Tt and 10% tt. Due to the small numbers in some of the genotypes (especially the ss genotype) it was not possible to apply statistical methods to the individual or combined genotypes. When Chi squared tests were applied to the numbers of patients bearing the S or s and the T or t allele in the control groups and osteoporotic groups there was no significant difference between them, whether they were classified by lumbar or hip T- or Z- score. One hundred females and 26 males had a history of fracture. The T allele was more prevalent in patients with a history of fracture (χ^2=5.05, p>0.02<0.05). This trend was evident in the steroid treated group (χ^2=5.65, p>0.01 <0.02), but not in the no steroid group (Figs. 1 & 2). There was no difference in the frequency of the S or s allele in patients with a history of fracture.

DISCUSSION

Osteoporosis is the most common metabolic disease. It is a disease in which the density or mass of the bone is reduced, leading to an increased risk of fracture. The ability to predict and prevent osteoporotic-related fractures would be of major benefit to both patient and NHS. Thus finding a biochemical or genetic marker which could predict those at greatest risk of developing osteoporosis is an attractive proposition. To date none of the biochemical markers have proved of any value in diagnosing osteoporosis, although they have their uses in monitoring the effects of treatment. The early reports on the COL1A1 Spl[2] and vitamin D receptor *Taq 1*[1] polymorphisms appeared hopeful of being able to correlate genotype with bone density and risk of fracture. However subsequent studies with both these polymorphisms have brought conflicting reports. In contrast to many previous studies [2, 4-7] we found no significant difference in the distribution of genotypes for the COL1A1 genotypes in the osteoporosis and control groups or any association between genotype and fracture. Other studies [8-11] report similar results to ourselves with no

Fig 1   VDR genotype in patients who had not been previously treated with corticosterolds
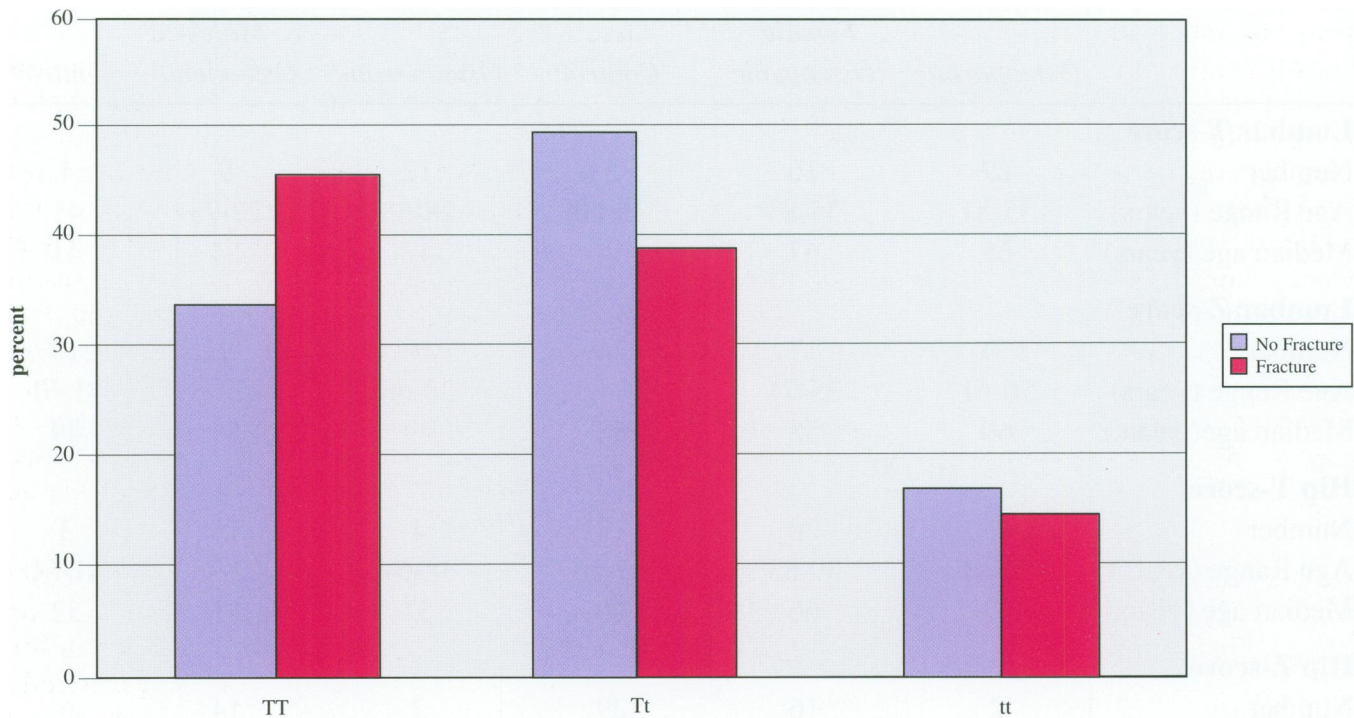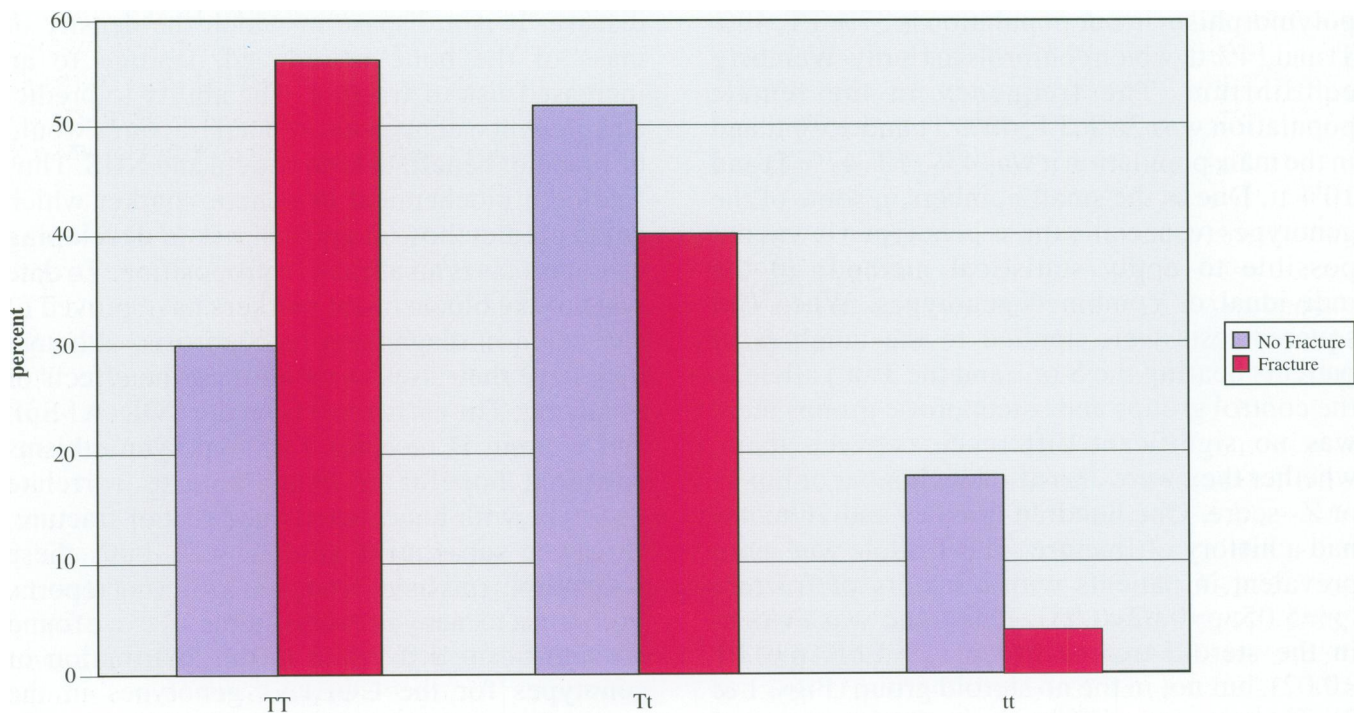

Fig 2   VDR genotype in patients treated with corticosteroids for other diseases

association between COL1A1 genotype and either BMD or fracture. Beaven[12] has reported ethnic differences in the prevalence of the s allele, being lower in an African and Asian population than in European countries and Han *et al*[13] have found the Spl polymorphism to be absent in a Korean population. However, all the studies mentioned above which found a positive association between the polymorphism and BMD or fracture risk were carried out with Caucasian European or American subjects, so racial differences are unlikely to be the reason for the disparity in the findings. In most cases the distribution of the genotypes was similar to ours in the non-osteoporotic population; it was in the osteoporotic group that the distribution of the genotypes differed in the studies which had positive findings. The criteria used to choose the patient group may be the key to the differences in the findings of the various studies. Utterlinden[14] whose overall distribution was very similar to ours, only found genotype related differences in the bone mineral density in women in the 75-80 year old age group, younger groups showed no difference between the genotypes. Since our study spanned a wide age range any differences in the oldest patients may have been diluted by the younger patients. (We did not pursue this possibility since, if genotyping is to be of any use in a clinical situation it would be very important that the risk of osteoporosis could be identified in young patients, before clinical osteoporosis is evident.) A recent study by Brown[15] found the association between COL1A1 polymorphism and the rate of lumbar spine bone loss to be dependent on dietary calcium. Carriers of the "s" allele lost more bone in the low calcium intake group but the carriers of the "S" allele lost more bone in those with a high calcium intake. Since our patients were chosen at random from patients attending the Osteoporosis Clinic it is likely that they spanned a range from low to high calcium intake.

We found no association between the *Taq 1* polymorphism genotype and BMD as assessed by both T-score and Z-score which again contrasts with some of the earlier studies" [1-6] but confirms other studies.[17-19] A meta- analysis of 16 published papers[20] showed great disparity between studies, with some finding an association between the TT genotype and lower BMD, some finding an association between the tt genotype and lower BMD and others finding no association. Marked racial difference in the distribution of the genotypes have been reported.[17, 21, 22] We found no over-representation of any genotype in either our steroid or no steroid groups with osteoporosis. However the TT genotype was over-represented in patients who had been treated with corticosteroids and who had sustained a low trauma fracture (56% in the fracture population compared to 30% in the control population). Most studies have excluded corticosteroid treated patients from their study population but one Australian study[23] which looked specifically at patients who were on corticosteroids for various diseases, like ourselves, failed to find any association between BMD and VDR genotypes at any site. A preliminary study by Chamberlain,[24] in a UK population, found an over-representation of the TT genotype in corticosteroid treated patients who had very low BMD or evidence of vertebral fracture.

Osteoporosis is a major side-effect of steroid therapy but some individuals do not develop osteoporosis. It is possible that the susceptibility to steroid induced osteoporsois is genetically modified. Glucocorticoids influence the regulation of calcium and phosphate metabolism and inhibit vitamin D_3-mediated induction of genes in osteoblasts [25] so polymorphisms or mutations in the vitamin D receptor may be one of the factors involved in how individuals handle their treatment. Our study suggests that the TT genotype may be a risk factor for fractures in patients treated with glucocorticoids. However 40% of patients with a fracture history were Tt and 4% were tt so further studies on large numbers of patients with steroid-induced osteoporosis are required.

Due to the small number of people with the ss genotype it was not feasible to compare people with the two "high risk" genotypes (ie ss and TT) with the seemingly "low risk" genotypes (ie SS and tt). One female had the ssTT genotype. Her lumbar and hip T-scores were −3.47 and −1.78 and Z-scores were −1.42 and −0.11 respectively, she had no familyhistory of osteoporosis but had had a low trauma fracture. The most frequent genotype in our population was SSTt (Table I) and this was consistent in all the groups of patients except the steroid treated patients with a history of fracture, in which SSTT was the most common genotype. This probably reflects our findings with the VDR receptor genotypes rather than a synergistic or additive effect of the two types of polymorphism. The number with the SsTt

genotype was considerably less in patients with fractures than those without fractures but the numbers were too small to draw any definite conclusions. Further investigation with larger numbers of patients may reveal more significant differences between the genotypes but for a genetic test to be of value in diagnosis or risk stratification one or two genotypes would have to stand out in the patient group, even in small numbers of patients.

In conclusion, we have been unable to present any evidence that either COL1A1 or VDR genotyping, singly or together, have a role in predicting either risk of osteoporosis or risk of fracture.

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